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MASTER'S THESIS

Thermoregulatory plasticity in flower spike pigmentation and reflectance in a northern population of *Plantago lanceolata*

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ABSTRACT

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Abstract:

In this study, a greenhouse experiment was used to assess if temperature sensitivity, specifically, thermoregulatory plasticity, has a functional role in floral reflectance and pigmentation in a population of *P. lanceolata* grown in three different temperature treatments, reflecting past, present, and future summer temperatures. Spectrophotometry, surface temperature readings, and near-infrared (NIR) region image analysis were used to identify how the spectral absorbance properties and biochemical makeup of *P. lanceolata* flowers differed between treatments. Reflectance and phenolic absorbance were both found to be influenced by ambient temperature. However, surface temperature of flower spikes was not affected by growing temperature, reflectance, or phenolic absorbance. The results suggest that *Plantago lanceolata* may utilize thermoregulatory plasticity in reflectance and phenolic absorbance to adjust to rising temperatures. These findings have important implications in species reactions to climate change and denotes that increased selection on thermal function traits may occur under a future climate scenario of continued warming in temperate and boreal biomes.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
g	Grams
HCA	Hydroxy-cinnamic acids
IR	Infrared
LM	Linear model
LMER	Linear mixed effects regression
NIR	Near-infrared
nm	Nanometers
RGB	Red, green, blue
ROS	Reactive oxygen species
UV	Ultraviolet

1. Introduction

1.1 Phenotypic plasticity and climate change

Global climate change impacts populations and individuals in a multitude of ways, including changes in physiology, growth patterns, fitness, behavior, or genetic and phenotypic composition (Reed et al. 2011). There are two ways through which adaptation to changing environmental conditions can occur: phenotypic plasticity and evolution by natural selection (Fox et al. 2019). Phenotypic plasticity, defined as one genotype having the capability to express several phenotypes depending on environmental circumstances (Chevin et al. 2010), largely accounts for species reactions to environmental variation and climate change (Scheiner 1993; Gienapp et al. 2008; Merilä and Hendry 2014; Bonamour et al. 2019). Phenotypic plasticity permits organisms to respond optimally to predictable environmental cues, which maximizes fitness (Piersma and van Gils 2011).

Phenotypic plasticity is often referred to as a "rapid-response mechanism", given that it acts on an individual level to produce a phenotype suitable for the ambient environment (Fox et al. 2019). Some species have already altered their phenology in response to climate change by adjusting the timing of life history events. For example, between 1973 and 2011, *Boechera stricta* populations in the Rocky Mountains began flowering significantly earlier in the season (Anderson et al. 2012). This change in flower phenology was strongly associated with warmer temperatures and earlier snowmelt dates (Anderson et al. 2012). However, most climate change studies that have investigated plant reproduction focused on reproductive timing rather than reproductive responses, such as thermoregulation, during a reproductive season (Lacey et al. 2010).

1.2 Thermoregulatory plasticity as a mechanism to cope with variation

Climate change will affect community interactions and selection pressures at both large scales and local scales. Local populations are expected to experience more extreme and variable weather patterns (Easterling 2000), which in turn could cause selection pressures to fluctuate and thus favor increased plasticity (Reed et al. 2011). Plasticity is positively associated with populations at higher latitudes, elevations, and experiencing seasonal temperature variation (Ghalambor et al. 2006). Lacey et al. (2010), for instance, found that reflectance and color plasticity of flower buds was positively correlated with latitude. Ectotherms, in general, exhibit direct relations between thermal plasticity and latitude and elevation (Ghalambor et al. 2006). Slight increases in flower temperature have been shown to augment reproductive success,

particularly in plants growing at high latitudes or elevations and those that bloom in early spring (Stanton and Galen 1989; Ida and Totland 2014; Distefano et al. 2018). Selection pressures for thermally plastic traits are thus, likely greater at higher latitudes and elevations.

There are several hypotheses that propose explanations as to why thermal plasticity is more adaptive at higher latitudes and elevations. Ghalambor et al. (2006) suggested that the magnitude of thermal variation is greater in higher latitudes and elevations, which explains increased thermal plasticity in said regions. Another similar explanation, known as the Magnitude Hypothesis, anticipated that plasticity is preferred to non-plasticity as environmental variability increases (Via 1993; Schlichting and Pigliucci 1998; Kingsolver and Huey 1998). The Frequency Hypothesis is yet another explanation, which states that thermal plasticity is more adaptive at higher latitudes and elevations due to thermally variable growing seasons, which are shorter, and cooler compared to populations growing at lower latitudes and elevations (Lacey et al. 2010). The Frequency Hypothesis is consistent with results from Lacey et al. (2010), and not consistent with the Magnitude Hypothesis. In this sense, thermal plasticity may rely on climatic variability, which increases with increased latitude and elevation.

Plantago lanceolata has been shown to express phenotypic plasticity in spike reflectance and color (Lacey and Herr 2005). It has been proposed that this plasticity in spike reflectance allows individuals to acclimate to differences in the temperature of their growing environment: this capacity is known as thermoregulatory plasticity (Lacey et al. 2010). *P. lanceolata* exhibits thermoregulatory plasticity in both the visible and near-infrared (NIR) regions (Anderson et al. 2013). Spike reflectance affects internal spike temperature when there is incoming solar radiation (Andersen et al. 2013). The rate of cellular respiration in *P. lanceolata* roots is limited when ambient temperature is below 15°C (Covey-Crump et al. 2002) or above 25°C (Atkin et al. 2005). Maintaining optimal flower temperature is imperative for reproductive processes to function properly within the flower, for instance, for embryo formation and abortion (Stephenson 1981). Therefore, selection favoring thermoregulation could have a great fitness benefit for processes such as metabolism and reproduction.

Another mechanism that underlies thermoregulatory plasticity in *P. lanceolata* spikes is phenolic compounds, primarily, anthocyanin content, which increases in colder temperatures (Stiles et al. 2007). Anthocyanins, which are widespread in angiosperms, are responsible for dark spike colors. Phenolic compounds carry out many biological functions in plants, mostly related to

interactions with their environment (Bautista et al. 2016). Several plant species exhibit increased anthocyanin levels in low temperatures (Rabino and Mancinelli 1986; Dela et al. 2003; Berdari et al. 2016). Moreover, total phenolic and antioxidant flavonoid content have been positively correlated with temperature (Bautista et al. 2016). Dark spike color leads to increased absorption, which helps warm reproductive tissues and thus contribute to higher fitness in cooler environments (Anderson et al. 2013). Flower pigmentation may adjust reflectance and flower temperature since dark flowers absorb more solar energy than light, reflective flowers (Jewell et al. 1994; Mckee and Richards 1998).

Fitness advantages, including offspring being produced earlier in the season when seed predation is lower (Lacey et al. 2003), is one advantage to producing dark spike colors. Moreover, their offspring will be more fit, as they too will have the advantage of floral warming (Lacey and Herr 2005). However, maintaining this reflective plasticity is also thought to be energetically costly due to the metabolic processes involved in synthesis of the pigments (Lacey and Herr 2005). All *P. lanceolata* genotypes are highly reflective at warm temperatures, but genotypes from lower latitudes do not show reduced reflectance at cooler temperatures. (Lacey et al. 2010). In other words, genotypes that experience cool temperatures, typically found in higher latitudes, are phenotypically plastic, while those at lower latitudes produce only highly reflective flowers and are thus non-plastic when it comes to thermoregulation.

P. lanceolata normally produces poorly reflecting, dark flowers during the spring and autumn when temperatures are lower, and reflective, light flowers during summer when temperatures are higher (Lacey et al. 2010). A study by Lacey et al. (2010) suggests that increased global temperatures will lead to decreased selection pressures favoring thermoregulatory plasticity at high latitudes and elevations. The effect of decreased plasticity of floral reflectance and color could potentially increase the incidence of non-plastic genotypes, which would change the genetic structure of current populations (Lacey et al. 2010). The links between reflectance and internal spike temperature suggest that thermoregulatory plasticity will influence the success of *P. lanceolata* in a changing climate. Existing research on how thermoregulatory plasticity will influence the success of populations at high latitudes and elevations has not been completely elucidated.

Understanding the role of thermoregulatory plasticity in reflectance and phenolic content has become a progressively important matter due to rapid climate change and the implications that

warming temperatures could have on plant reproductive systems. Better understanding thermoregulatory plasticity in a model organism such as *P. lanceolata* could benefit future plasticity studies and moreover, the understanding of species reactions to climate change. A study specifically focused on a high-latitude, thus likely plastic population of *P. lanceolata* would provide an opportunity to study the extent of thermoregulatory plasticity in this species.

1.3 The study plan

The aim of this thesis is to examine thermoregulatory plasticity in a northern population (occurring at 60.2°N) of *P. lanceolata.* By assessing the degree of thermoregulation plasticity of this population, we can improve our understanding of how the population might be affected by predicted future temperatures. A previous study has already found a link between anthocyanin content and ambient temperature among high-latitude populations (latitudinal degrees: 61.1°N of *P. lanceolata*) (Lacey et al. 2010), so a similar pattern was expected. Plasticity in reflectance and color increases as latitude increases (Lacey et al. 2010). However, it remains unclear whether anthocyanin content is related to floral pigmentation and further to the temperature of flowers. Thus, studying a population originating from a northern *P. lanceolata* population provides a good opportunity to understand how the mechanisms work. Many studies (Lacey and Herr 2005; Stiles et al. 2007; Lacey et al. 2010; Anderson et al. 2013) also found indications of a connection between reflectance and ambient temperature. Thus, in this study, I expect the data to show thermoregulatory plasticity owing to reflectance and phenolic content in this northern population of *P. lanceolata.* In Lacey et al. (2010), source populations of *P. lanceolata* ranged from 39.3°N to 61.1°N, and the populations at higher latitudes showed more thermoregulatory plasticity. Also, both increased reflectance and darker color were positively and significantly correlated with higher latitude (Lacey and Herr 2005). Stiles et al. (2007) provided insight into temperature regulation of anthocyanin production by determining that darker *P. lanceolata* flower bracts are produced in cooler temperatures in a mid-latitude population. However, the same type of study has not been implemented in a northern population, and thus temperature regulation of anthocyanin production in a northern population of *P. lanceolata* would provide more insight into thermoregulatory plasticity and phenolic compounds produced in northern populations. Hence, a population that occurs at 60.2°N was chosen for this study, which would likely show high plasticity in flower color and reflectance, thereby enabling tests of phenolic absorbance, reflectance, and surface temperature in a northern population.

To determine the role of reflectance and pigmentation in enabling thermoregulation of a northern population of *P. lanceolata*, the reflectance, phenolic content, and external temperature of their flowers were studied. Also, to determine if spike size varied with temperature, the relationship between temperature and floral spike dry weight was examined. If phenolic compounds have a mechanistic role in thermoregulatory plasticity, spikes grown in cooler environments would be expected to contain more anthocyanins and other phenolics. Additionally, the reflectance in the infrared (IR) region of floral bracts was expected to increase as growing temperature increased. Lastly, increased spike absorbance in the IR region and phenolic absorbance was anticipated to increase surface flower temperature. The following hypotheses were tested: 1) phenolic content is higher in floral bracts from plants grown in cooler environments (Figure 1.A-B); 2) reflectance of floral bracts is higher in plants grown in warmer environments (Figure 1.C-D); 3) as flower bract absorbance of radiation and phenolic absorbance increase, spike temperature increases (Figure 1.E-F).

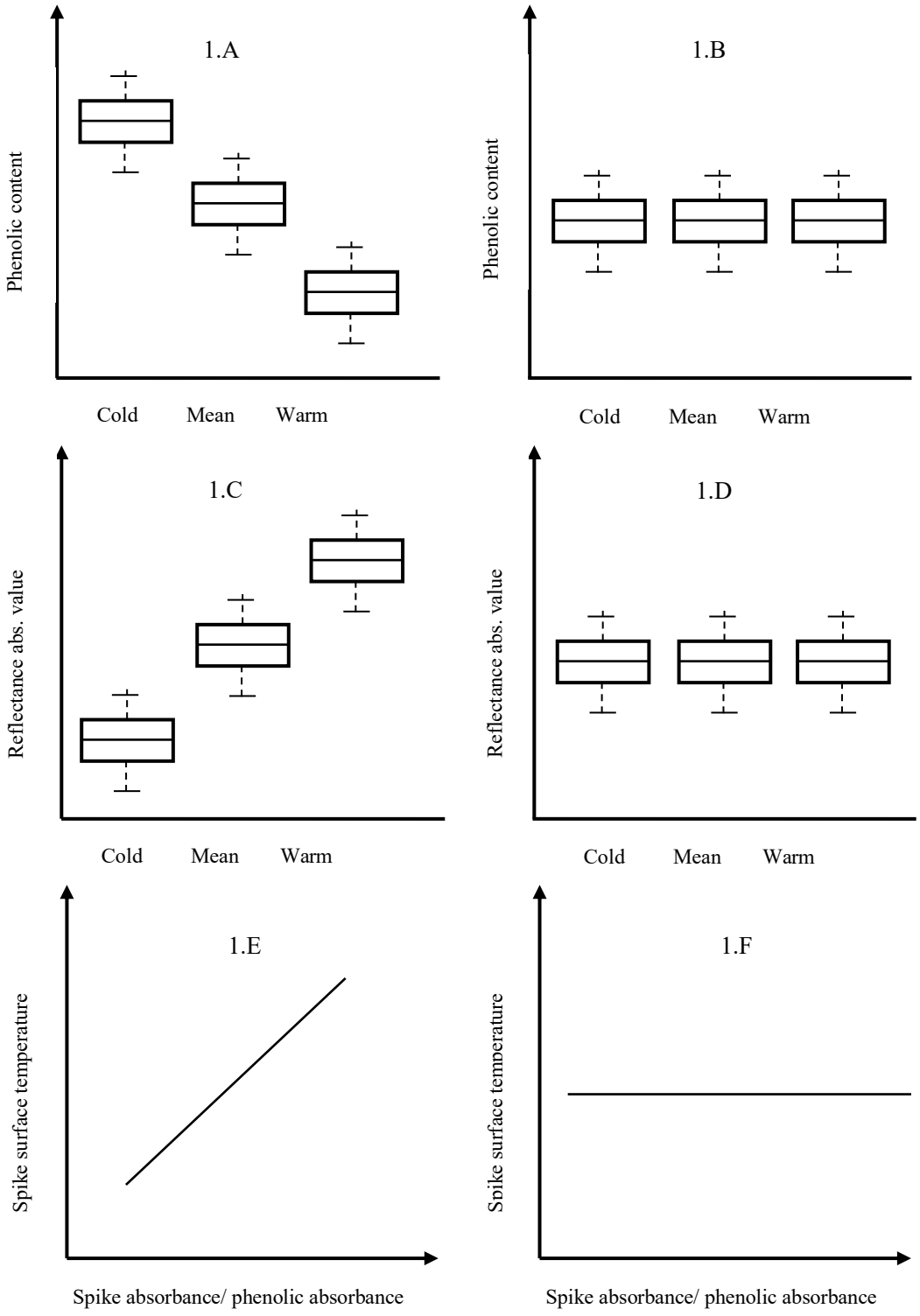

Figure 1. A. Hypothesis 1 and expectations for experiment; B. Null hypothesis 1; C. Hypothesis 2 and expectations for experiment; D. Null hypothesis 2; E. Hypothesis 3 and expectations for experiment; F. Null hypothesis 3.

2. Materials and Methods

2.1 Plant material

Fresh *Plantago lanceolata* L. (Plantaginaceae), commonly known as ribwort plantain or English plantain, is a perennial herb native to Eurasia that is found in disturbed sites. Plants grow vegetatively as rosettes and produce long-stalked spikes (inflorescences) from leaf axils (Lacey and Herr 2005). *P. lanceolata* seeds were collected from up to 50 individuals from nine populations from Åland (southwestern Finland, c. 60°N 20°E, see appendix; Table S1) in August 2019. In Åland, *P. lanceolata* grows in meadows, pastures, and rocky coastal areas (Opedal et al. 2020).

2.2 Experimental Design

Seeds were germinated at the Viikki Greenhouse and Field Station, University of Helsinki, Finland during Autumn, 2019. The plants were grown in six greenhouse compartments representing three temperature treatments with two replicates of each treatment. Seeds were collected from nine populations, and ten mothers from each population were sown. Twelve seeds were germinated from each mother and randomly divided among three treatments and two replicates for each treatment, for a total number of 1,296 seeds sown. 523 germinated seedlings were included in the experiment. For every treatment, two individuals from the same mother were chosen, with one being placed in each of the two replicated greenhouse chambers of the same temperature (Figure 2). Altogether, between 82-91 individuals were grown in each replicate.

Each treatment was a different temperature, which was chosen using climate data from the Åland islands (1959-2018; Figure 3). They are loosely based on observed average temperatures in June and July in Åland during 1959-2018, focused mainly on July temperatures. The minimum average maximum temperature for July had been about 17°C, which represented the cold treatment temperature during the day. The mean treatment temperature of 20°C was decided by taking the mean across this time-period. The warm treatment temperature was set at 23°C so that the difference between each treatment temperature would be the same. The same method was applied to choosing the night temperatures, which each

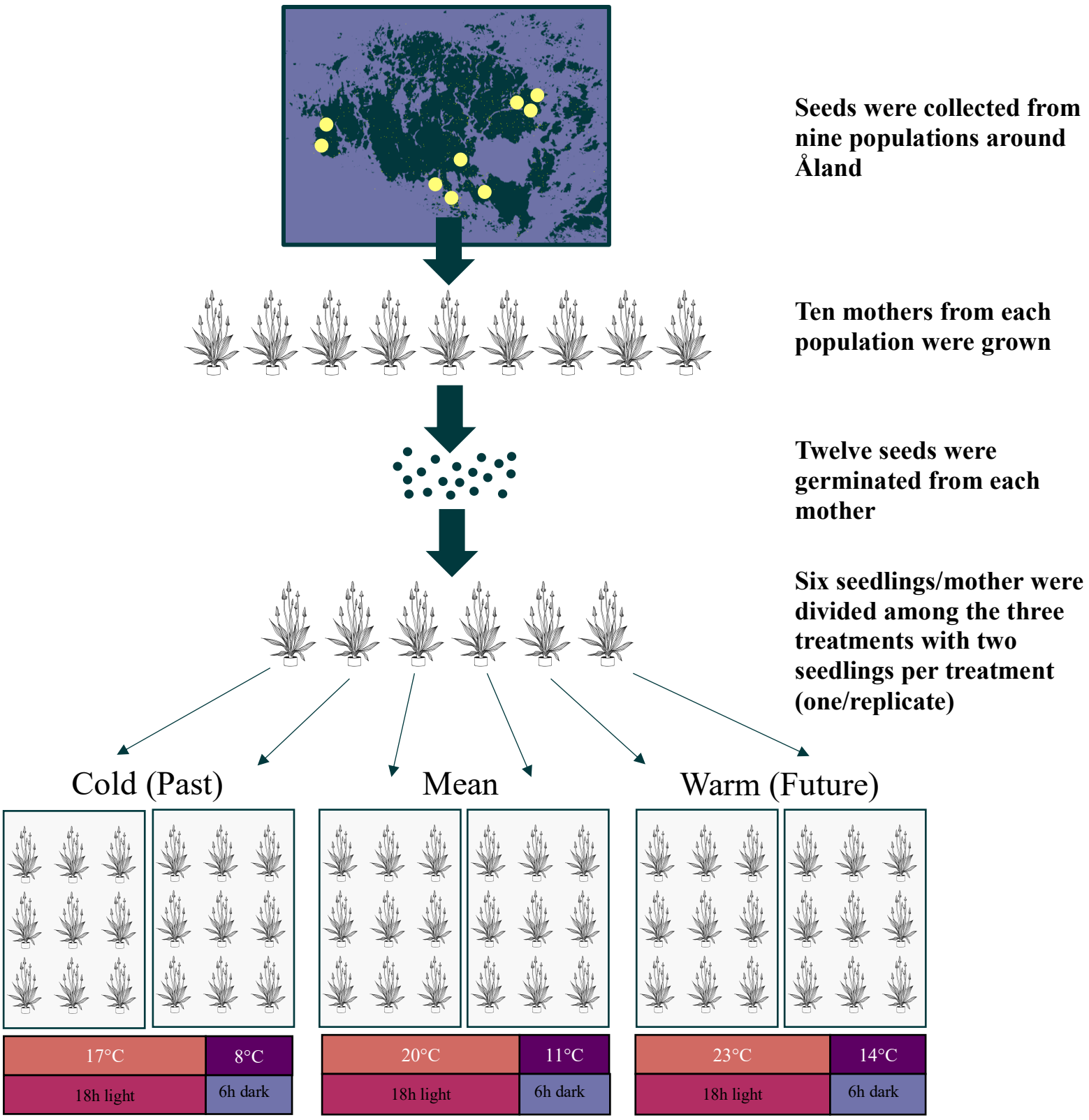

Figure 2. Experimental design.

had a 3°C difference between treatments. The three temperature treatments were: Cold (17°C during the day and 8°C at night); Mean (20°C during the day and 11°C at night); and Warm (23°C during the day and 14°C at night). Temperature and humidity of each replicate room were monitored (see Figure 4 for mean room temperatures over a 24-hour period; see appendix S1-3 for daily room temperatures, daily humidity, and humidity over a 24-hour period). The same lighting conditions (18 hours of light and 6 hours of dark) were used for all treatments.

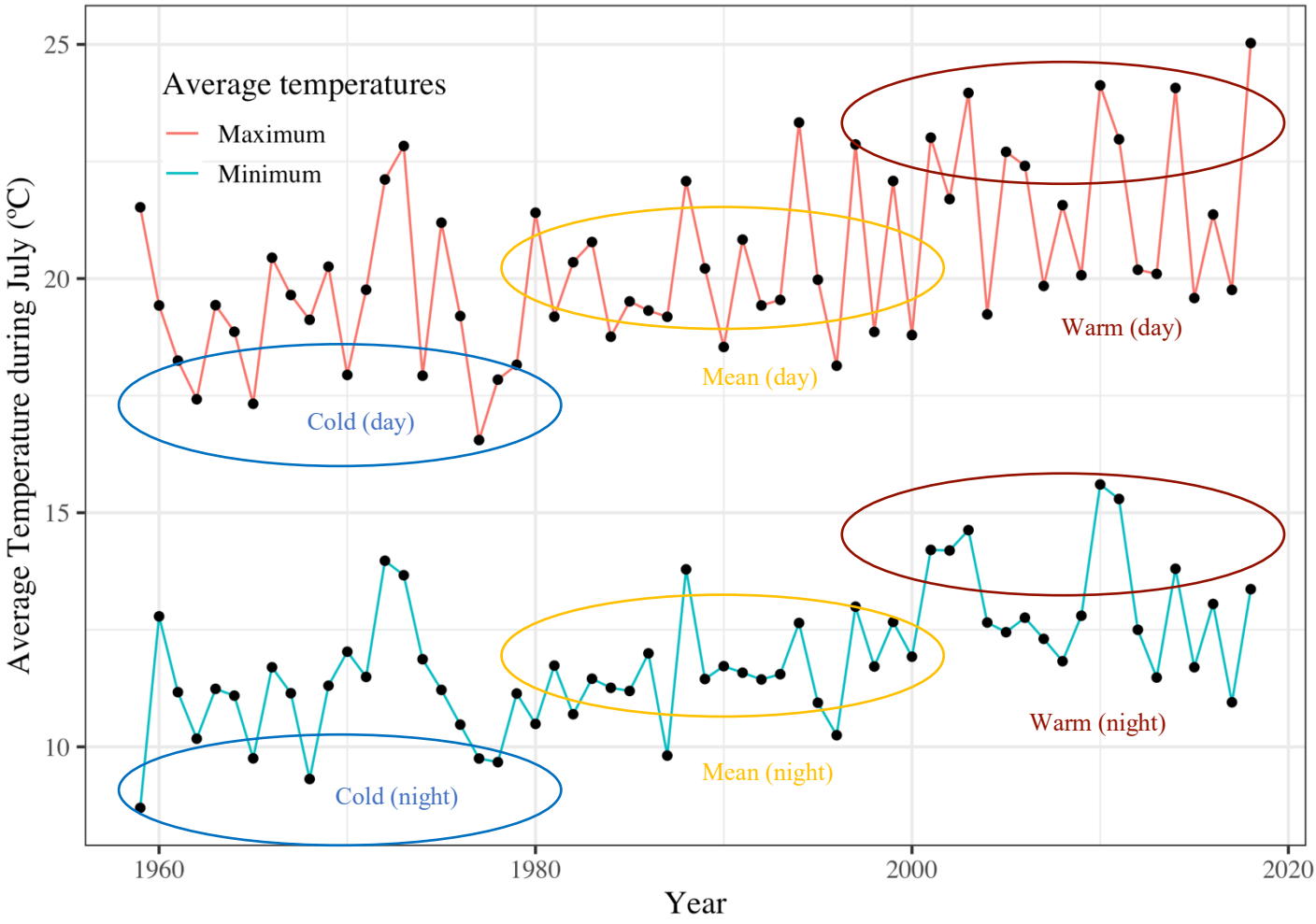

Figure 3. Maximum and minimum average temperatures in Åland during July from 1959-2018.

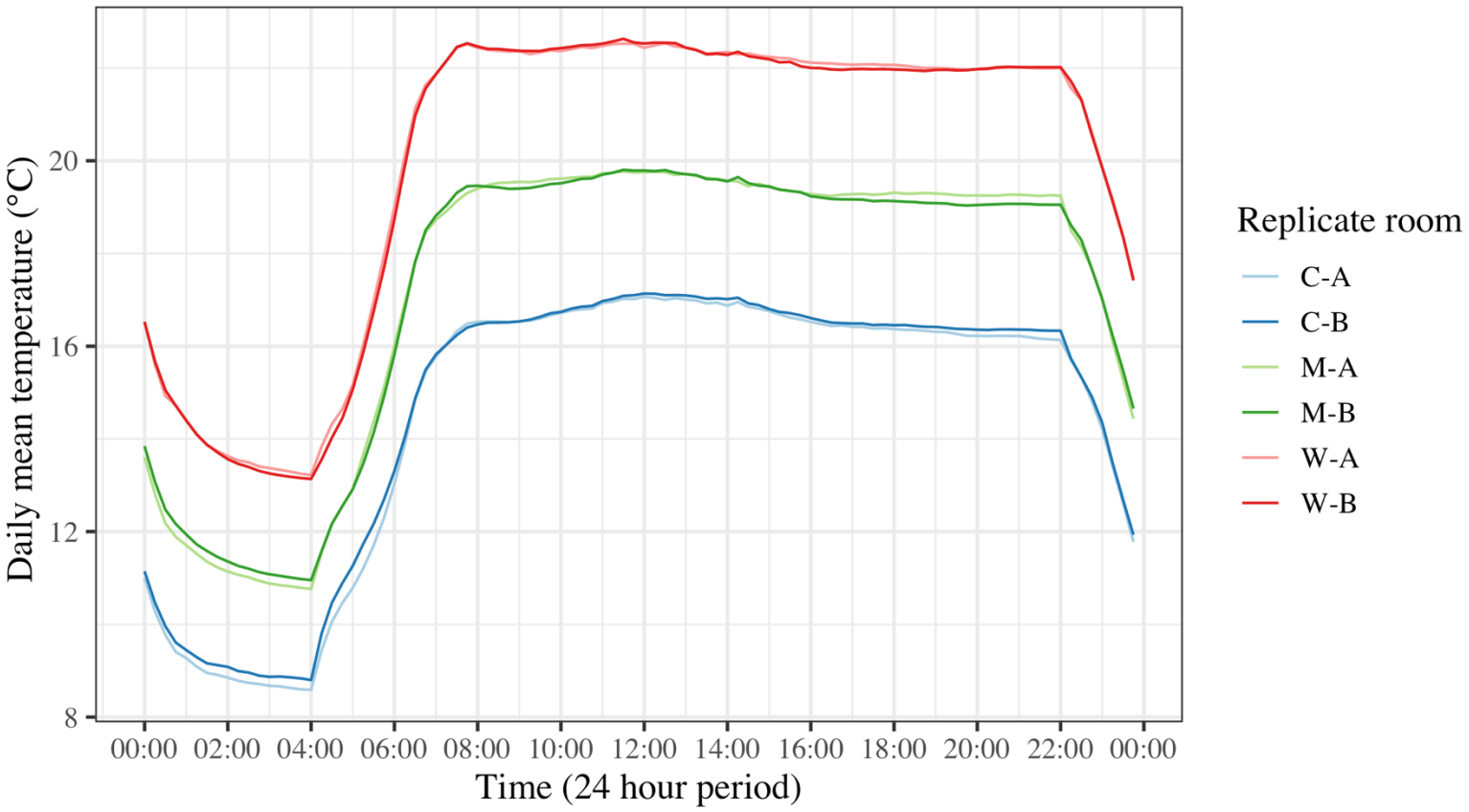

Figure 4. Mean replicate room temperatures (°C) over a 24-hour period, taken at 15-minute intervals from 17.09.2019 - 19.02.2020.

Seeds were sown directly into treatment conditions in Viikki greenhouses from September 17-20, 2019 and were checked for germination for the following two weeks. On October 9th, plants to be used in the experiment were re-potted. On October 16th, 13 plants omitted in the first potting round were re-potted. The potting mix contained four parts soil, two parts perlite, one part fine sand, and one part course sand. Plants were placed on tables with a watering rug (Image 1) and watered for three minutes every two days by inundating the tables. Each room had two rows of lights. On October 29th, plants were rotated (one column on left moved to the right of the group; one top row lowered to the bottom row of the group). Plants were thereafter rotated on a weekly basis. The first leaf measurements were taken from October 1st to November 2nd. On November 14th, the watering regime was changed to three minutes every three days, as there is less sunshine that affects the evapotranspiration during winter. At this point, plants were also rearranged into a new staggered pattern, which allowed more space between pots (Image 1).

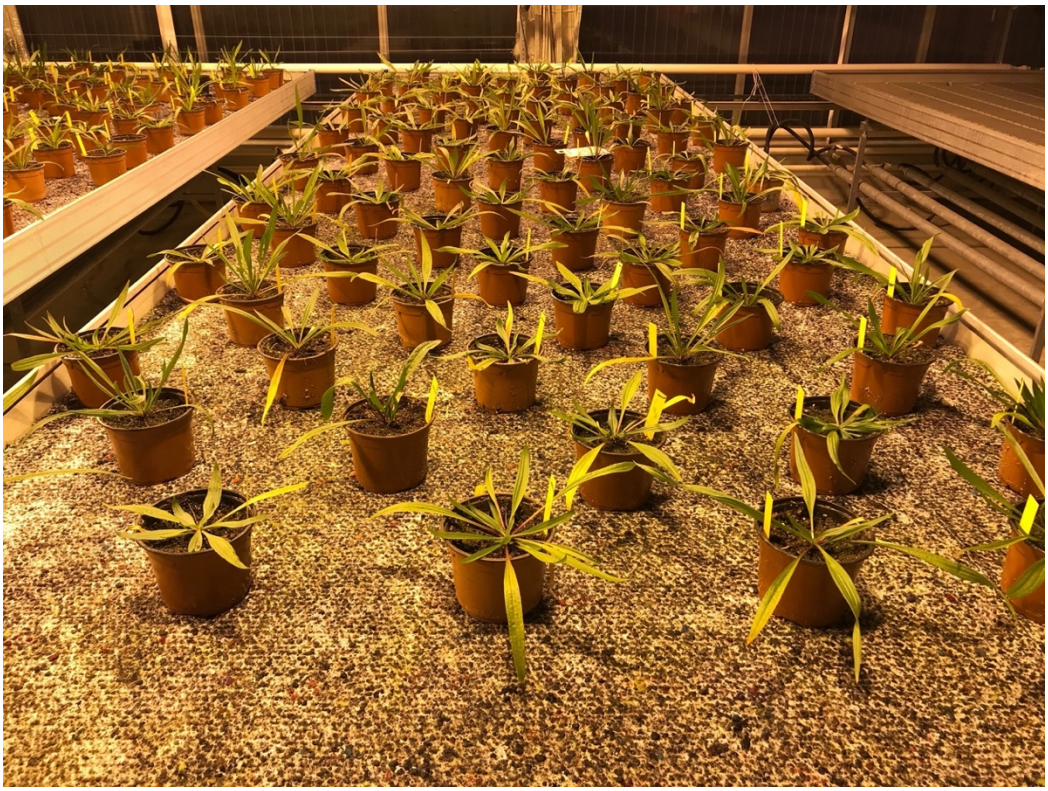

Image 1. Greenhouse setup example of staggered pattern used during majority of experiment.

On November 22nd, plants were fertilized (using Kekkilä Professional Surepex NPK 12-5-27: 2kg/1000 L= 20g/10 L bucket, with 5 x 10 L buckets per table). Plants were bathed in the fertilizer for at least 30 minutes after which the tables were drained. On the same day, Nematode application was applied on soil, using 1 teaspoon of nematodes in 3 L of water. The mixture was combined well and applied to soil. Two species of entomopathogenic nematodes ordered from Biotus Oy were used: *Steinernema feltidae* to control sciarid flies and *Steinernema carpocapsae* to control some species of flies, moth larvae, and weevils. The application was done after the fertilization and automatic watering; thus, the soil was humid. It is important to note that nematodes are highly sensitive to desiccation and ultraviolet (UV) light. Lights were on when the application was done, but it was done at the end of the day. Two factors may have prevented an optimal nematode effect: exposure to UV light and the difficulty to evenly dilute the nematode paste in water.

On January 13th, 2020, plants were again fertilized using the same methods described above. Nematode application was also done on the same day, using the same methods described above.

Plants were also fertilized using the same methods on January 30th, February 5th, February 12th, and February 19th. Plants were fertilized frequently during these weeks due to flower spikes being collected during this time. Starting January 30th, mature spikes were collected. Spikes were collected one to three times per week, based on availability of spikes. Flower spikes were collected before stigmas, flower petals, and anthers emerged from the sepals and subtending bracts (see Image 2). Not all spikes were collected due to the short time frame between budding and flowering. The last collection event occurred on February 18th, at which point spikes from 317 unique plants had been collected (Table 1).

Table 1. Number of plants collected from in each treatment temperature.

Cold	**Mean**	**Warm**
72	115	130

Image 2. Development stage of flower spikes when collected.

2.3 Infra-red thermometer reading and image capture in the IR spectra

On the same day that spikes were collected, they were brought to a photography room in Biocentre 3 on Viikki Campus, where their surface temperature was measured using the Optris LS Portable Infrared Thermometer. Spikes were placed on a white surface under a lamp with an incandescent bulb (for spectra see appendix: Figure S4) for 30 seconds, followed by the temperature reading being recorded. The lamp was positioned at a 45° angle toward the spike. There was 40 cm distance between the bulb and the spike (see Image 3). This allowed any differences in the absorbance and optical properties of the spikes to be recorded.

Similar studies measuring reflectance in P. *lanceolata* showed that temperature affects reflectance/absorbance mostly in the visible (525-650 nm) and NIR (725-850 nm) regions (Lacey and Herr 2005). Thus, photos were taken in the NIR region to get a better visual of reflectance in the floral bracts.

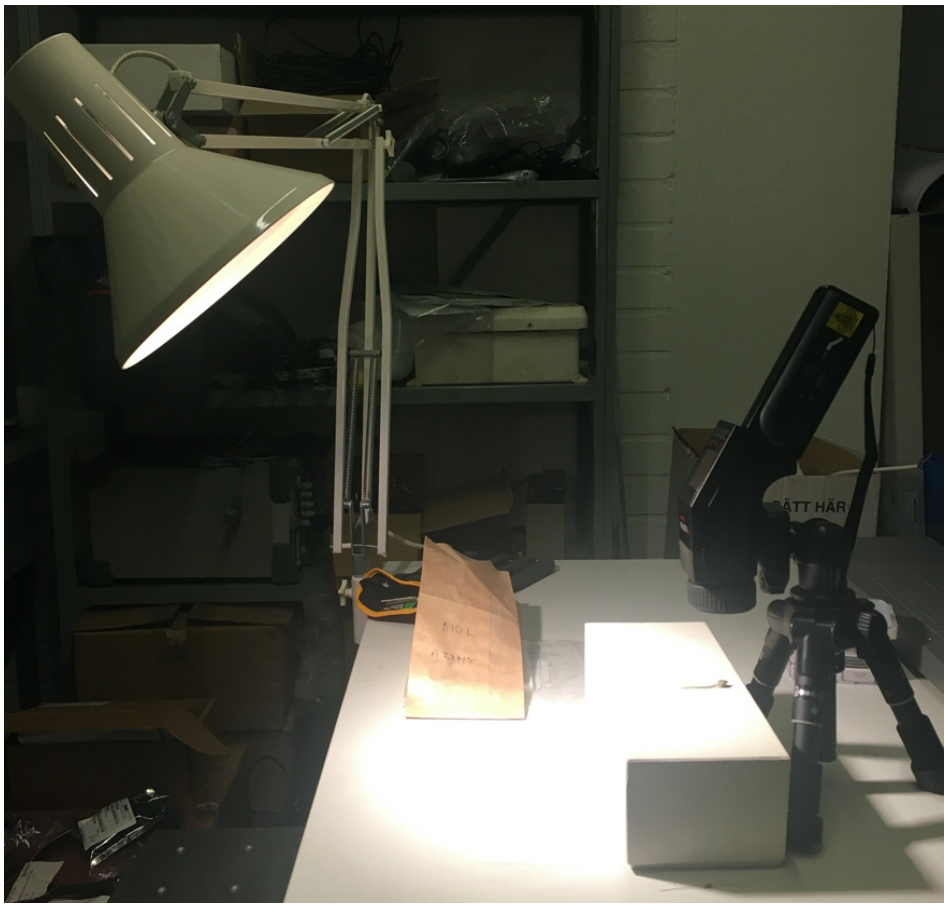

Image 3. Experimental setup for measuring spike surface temperature.

IR photographs were taken of the same spikes used for the IR temperature reading. Images were captured using an Olympus E-M1 camera under manual exposure. The camera was converted to full spectrum by replacement of the sensor's built-in UVIR-cut filter by quartz glass. The conversion was done by DSLR AstroTEC. IR photos were taken with a Sigma 30mm F2.8 DN lens and a 780 nm Heliopan RG780 ES 52 filter (for spectra see appendix: Figure S5), which attenuates light below 780 nm. Lights in the room were turned off and a lamp with an incandescent bulb (for spectra see appendix: Figure S4) was used for a light source. Comparison of these photos among flowers allowed any differences in absorbance characteristics to be distinguished.

IR photos were later processed using RawDigger: Research edition (Version 1.4.2). RGB channel sensor counts were assessed by taking two samples from each spike, each 300x300 squares at the tip and the widest part of the spike (see Image 4).

Image 4. Example of IR photo analysis, including size of 300x300 squares used to measure channel sensor counts.

A white Teflon slab with 95% reflectance was used as a white reference in each image, and the following equation was applied to the sensor counts, which then calculated percent reflection in the NIR region, which was then normalized across samples:

*((counts for spike) / (counts for Teflon slab / 0.95)) *100*

2.4 Drying and weighing of flower spikes

After photographic analysis, flower spikes were stored at room temperature with silica gel for two months. Prior to extraction and analysis, flower spikes were dried in drying racks for 48 hours at 45°C. Once spikes were completely dried, excess stems were cut off and spikes were weighed using a GWB PB303 DeltaRange scale.

2.5 Spectrophotometry

Spikes were sampled and preserved for spectrophotometry analysis (using methods described in Esteban et al. 2009), to determine phenolic content. The four bottom-most bracts were plucked from each spike for analysis. Due to limited time, only one spike from each unique individual was selected for spectrophotometry. The four bracts were combined with 8 µl of 100% acidified methanol and incubated for 48 hours at 4°C. The absorbance of extracts was assessed via spectrophotometry, and those samples with high absorbance peaks were diluted and remeasured and adjusted for the dilution factor to obtain accurate absorbance values. A Hellma Quartz cuvette (Type No. 104-QS) was used for spectrophotometric analysis of the methanol extracts. Absorbance was measured using a Shimadzu UV-2501 PC UV–VIS Spectrophotometer (Kyoto, Japan). Methanol blanks were measured between every ten samples for calibration. Once the spectra (190-700 nm) were produced, peaks and troughs were identified and peak sizes per nm were calculated and adjusted for dilution by multiplying the diluted samples up to the equivalent concentration of the undiluted samples. The ratio between peaks, specifically 291 nm to 331 nm, was then calculated to account for differences in the absolute values related to dilution of different sizes of flower bracts.

2.6 Data Analysis

The effect of treatment temperature on reflectance, phenolic absorbance, surface temperature, and dry weight of spikes was tested with a linear mixed effects regression (LMER) through the lmer function as implemented in the lme4 R-package (Bates et al. 2015). I assumed non-independence between population (origin population of plant) and the room in which plants were grown (replicate), whereby mixed effects models were fitted with both these variables as random effects factors. Due to the low number of spikes produced in colder temperatures, the dataset was small in the cold treatment (n = 72) and missing values for one level of population often caused a singular fit of the models. For this reason, every model was first tested with both

replicate and population as random effects, and if there was a singular fit issue, the data was separated so that observations from room (replicate) "A" were fitted separately from observations from room "B". The models were then compared to determine if the produced estimates differed between the models, and thus whether the room as a random effect could be omitted. If population caused a singular fit issue, a model with population as a fixed effect was compared to a model without population to determine whether population had a significant effect on model fit (as suggested by Robert LaBudde in Bolker (2021)).

The main hypotheses (Figure 1) were tested with four separate models. Hypothesis 1 postulated that the ratio of phenolic absorbance was higher in flower bracts from plants grown in cooler environments. Thus, the effect of treatment temperature on flavonoid and cinnamic acid major peak ratios (291 nm to 331 nm) was tested (in a subset of 317 plants) using a LMER. The phenolic absorbance ratio was not normally distributed, so it was transformed using a Box-Cox power transformation. Hypothesis 2 suggested that reflectance of flower bracts was higher from plants grown in warmer environments. Thus, the effect of treatment temperature on reflectance was tested (in the same subset of plants) using a LMER. Additionally, flower spikes were expected to be heavier in warmer temperatures, so the effect of flower spike dry weight on treatment temperature was tested (in a subset of 119 plants) using a LMER.

Hypothesis 3 proposed that as flower bract absorption of solar radiation increased, spike surface temperature would increase, and surface temperature of spikes would be higher in spikes with greater phenolic absorbance. Therefore, the effect of flower bract absorbance/reflectance (n = 123) and phenolic absorbance (n = 134) on surface temperature was tested using two separate LMER. To rule out the effect of treatment temperature, the models were also ran including this variable, which did not improve model fit in either model (Both models: $\chi 2$ =1.34, df=2, p=0.51).

In the model where phenolic absorbance was the response variable, the estimates of the models with data from the "A" versus "B" rooms did not differ substantially ("A" Estimates = 0.940 ± 0.027 (cold), 0.911 ± 0.032 (mean), 0.862 ± 0.032 (warm); "B" Estimates = 0.942 ± 0.027 (cold), 0.904 ± 0.027 (mean), 0.878 ± 0.030 (warm)). For this reason, it was left out of the model on which inference was based. Also, in the model where phenolic absorbance was the response variable, population "A" caused a singular fit of the model, so the estimates from the model including population "A" were compared with the estimates of the model excluding population

"A"- there was no significant difference in the outcome of the two models compared. For this reason, population "A" was removed from the model based on which results are presented in this study. In the model where dry weight of floral spikes was the response variable, population was not significant as a fixed effect ($\chi 2 = 4.51$, df: 8, *p*-value = 0.89), so it was excluded as a random effect from the model. See Table S2 in the appendix for the full model designs.

To calculate the statistical significance of predictor variables (treatment temperature, reflectance in the IR region, or phenolic absorbance), likelihood ratio tests (ANOVA) of the full model were assessed against intercept-only models, that is, a model without the fixed effect treatment temperature, for example, was compared to the full model using ANOVA.

The assumptions of the models were verified by visually checking plots of the residuals against each response and explanatory variable, and the distribution (histogram) and QQ plots of the residuals. All patterns indicated normally distributed residuals with no signs of heteroscedasticity nor extreme outliers. All data management and analyses were conducted in R studio (R version 1.2.5033 for Mac; (R Core Team (2019)).

3. Results

3.1 Reflectance and phenolic content across each treatment

Reflectance of flower bracts was found to be affected by treatment temperature, with highest reflectance in the warm treatment, followed by the mean and cold treatments (Estimate = 0.50 ± 0.031 (cold), 0.59 ± 0.041 (mean), 0.60 ± 0.042 (warm); Figure 5). This effect was evidenced by the better fit for the model including treatment temperature compared to the intercept-only model (Table 2). The ratio of phenolic compounds at 291 nm and 331 nm was determined to be highest in the cold treatment, followed by the mean and warm treatments (Estimate = 0.94 ± 0.016 (cold), 0.91 ± 0.020 (mean), 0.87 ± 0.020 (warm); Figure 6). This result was determined by the better fit for the model including treatment temperature compared to the intercept-only model (Table 2).

3.2 Effects of phenolic content and reflectance on surface temperature of floral bracts

Reflectance in the NIR region (Estimate = 21.10 ± 0.389; Figure 7) nor absorbance of phenolic compounds (Estimate = 20.83 ± 0.220) were affected by surface temperature. This effect

was supported by the better fit for the intercept-only models compared to the models including surface temperature of floral bracts (Table 2).

3.3 Effect of temperature on floral spike weight

Floral spike dry weight was found to be affected by treatment temperature, with the heaviest weight in the warm treatment, followed by the mean and cold treatments (Estimate = 0.023 ± 0.002 (warm), 0.021 ± 0.002 (mean), 0.017 ± 0.001 (cold); Figure 8). This effect was supported by the better fit for the intercept-only model compared to the model including treatment temperature (Table 2).

3.4 Spectrophotometry results

The spectra produced from the spectrophotometry of methanol extracts of floral bracts revealed seven major peaks of absorbance, which corresponds to the sum of absorbance by classes of compound present at each wavelength. Five peaks were in the UV region of the spectrum, and two peaks were in the visible region of the spectrum. The major peaks and estimates of compounds in each peak are summarized in Table 3. When visually comparing the mean relative values of absorbance rather than the peak ratios, the warm treatment shows greater absorbance compared to the mean and warm treatment (see Figure 9 for mean spectral absorbance for each treatment).

Table 2. Change in deviance for each model compared to an intercept-only model.

Variable (*y*~*x*)	**Change in deviance (χ2)/ Sum of squares**	**df**	***p*-value**
Reflectance~ Treatment temperature	7.12	2	**0.01**
Phenolic absorbance (291:331 nm)~ Treatment temperature	12.79	2	**0.001**
Surface temperature of spike~ Phenolic absorbance (291:331 nm)	0.021	1	0.88
Surface temperature of spike~ Reflectance	0.32	1	0.57
Dry weight of spike~ Treatment temperature	9.14	2	**0.01**

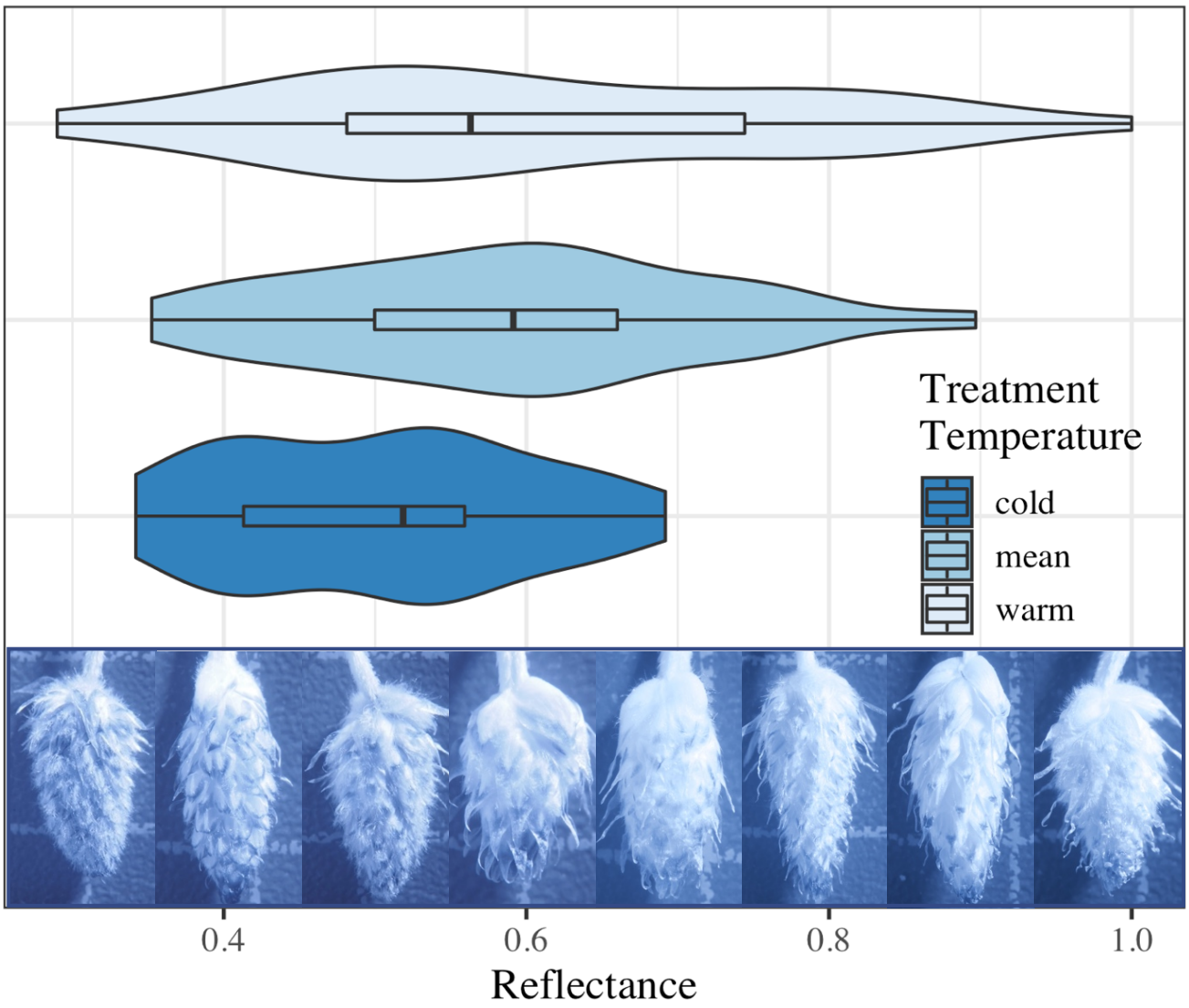

Figure 5. Violin plot showing reflectance in each treatment, with reference photos at the bottom showing the scale of reflectance values from IR photos (780 nm).

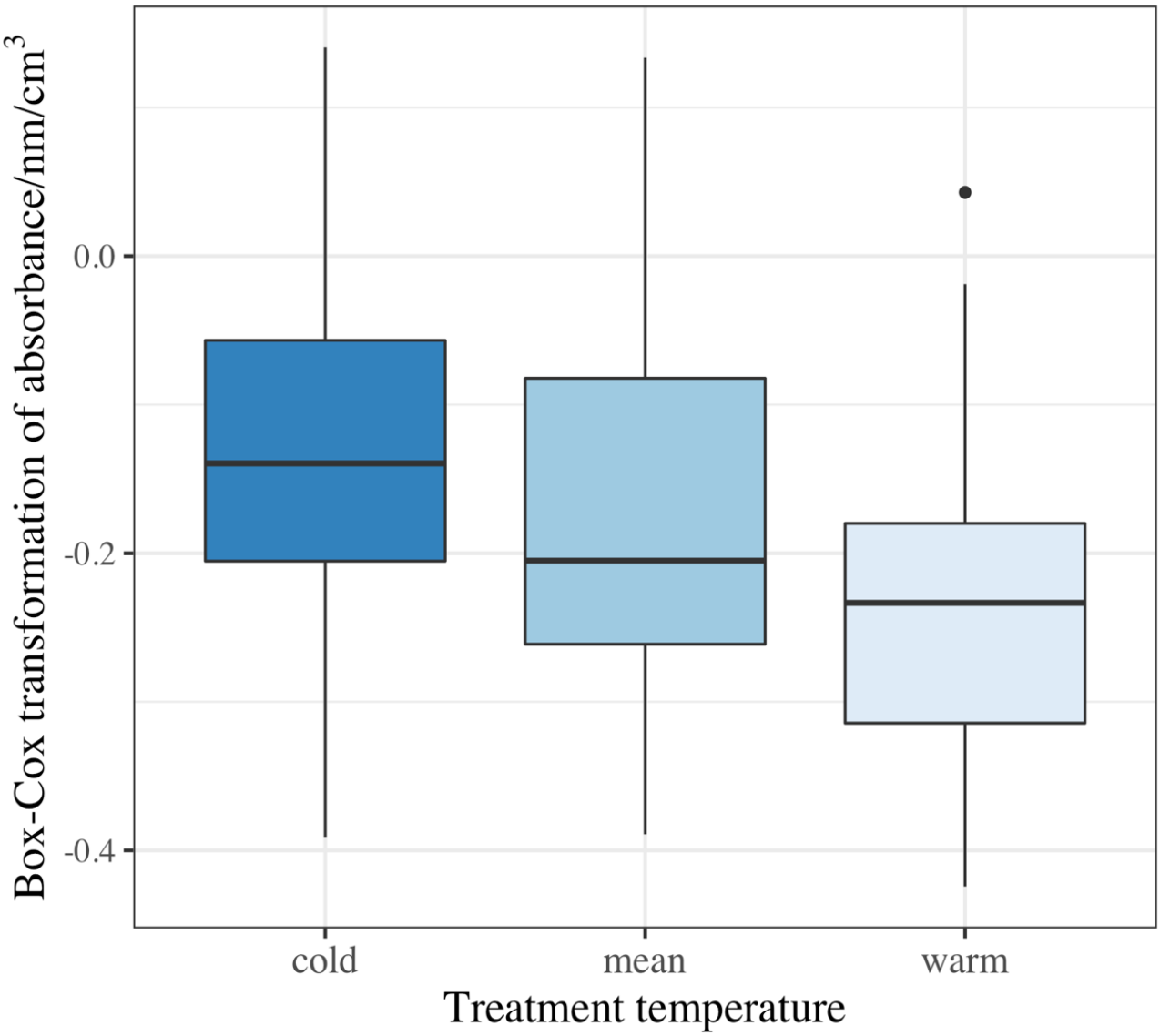

Figure 6. Boxplot of phenolic absorbance/nm/cm^3 (transformed with a Box-Cox power transformation) for peak ratio 291 nm to 331 nm in each treatment temperature.

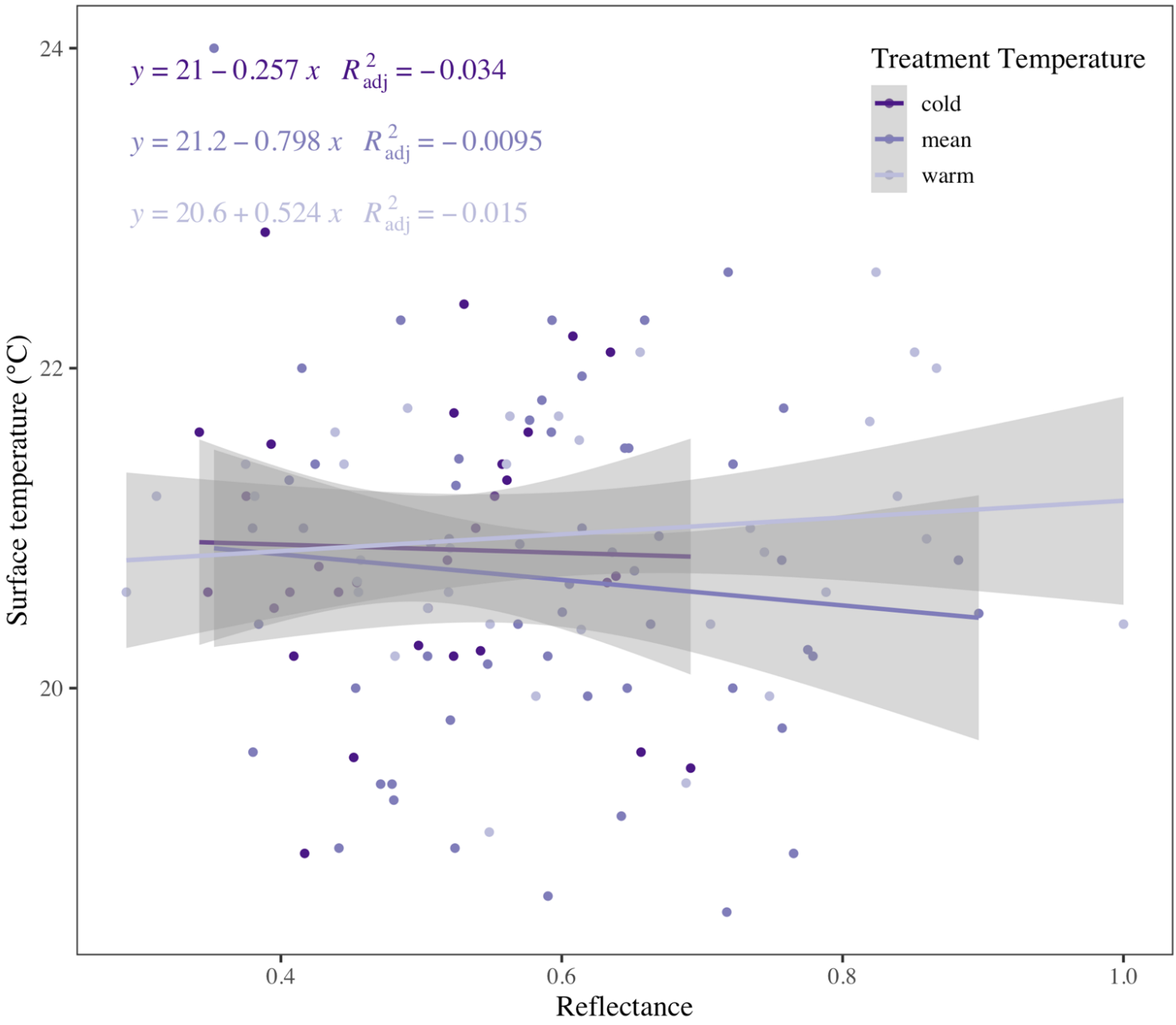

Figure 7. Relationship between surface temperature and reflectance at 780 nm to 1000 nm, fit with a linear regression model.

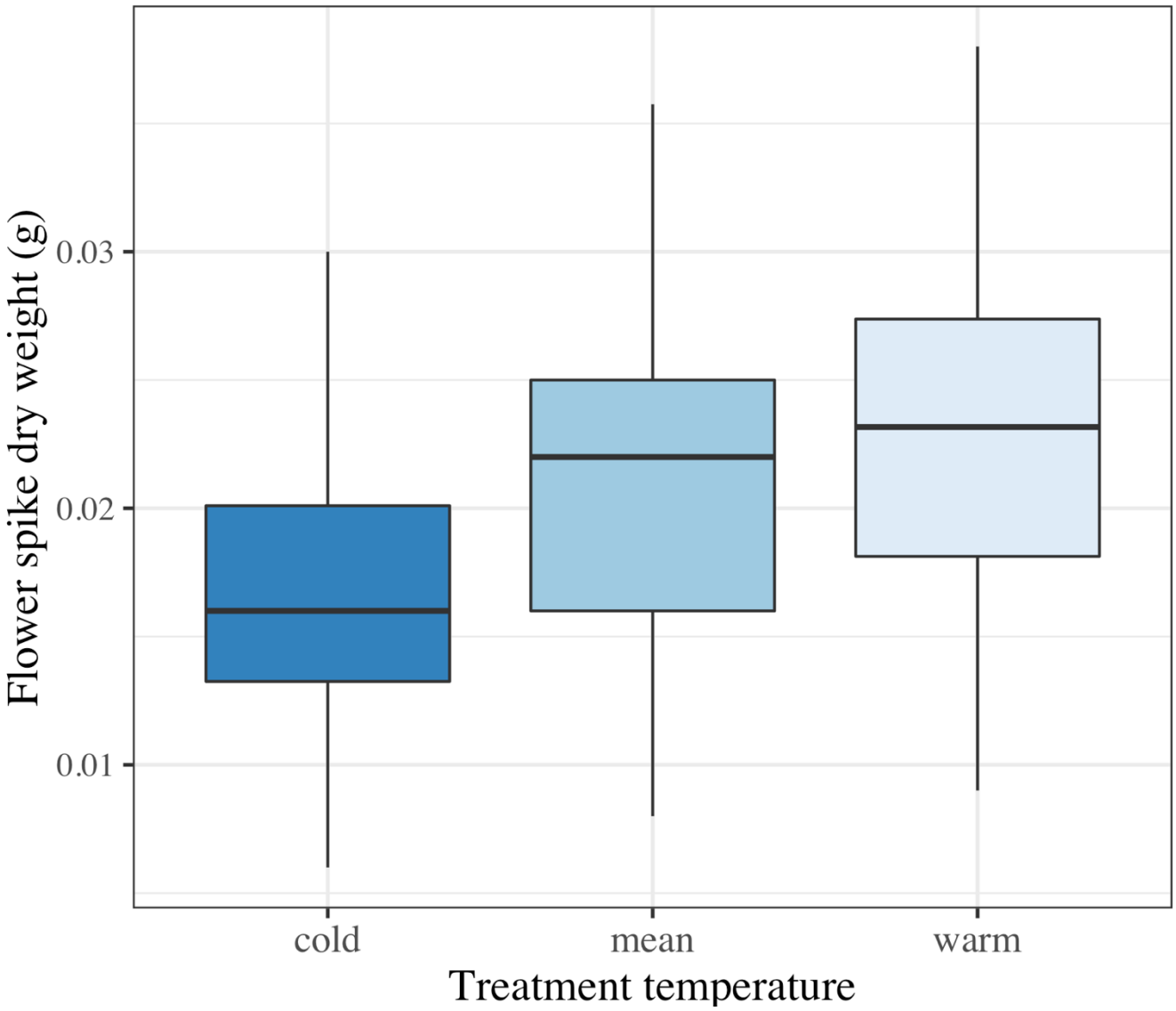

Figure 8. Boxplot of flower spike dry weight (g) in each treatment temperature.

Table 3. Major absorbance peaks with best guesses of compounds present at each peak. Best guesses came from my email correspondence with Jakub Nezval from the University of Ostrava.

Major absorbance peaks (nm)	**Region of spectra**	**Best guess**
204	UV	Primary metabolites and methanol
221	UV	Primary metabolites and methanol
252	UV	Flavonoid A-ring from, e.g., flavan-3-ols (quercetin, kaempferol) and flavones (apigenin, luteolin), probably glycosylated since this shifts the absorbance band more in the UV direction compared to flavonoid aglycones.
291	UV	Likely to be HCA's (ferulic acid, chlorogenic acid or their derivatives); there is the shoulder of their main absorption band. If due to the presence of cyanidin, one should also see its band somewhere about 255nm.
331	UV	According to the position of this main UV-A band, it is likely that flavan based derivatives (apigenin, luteolin) could be more abundant than flavan-3-ols (quercetin, kaempferol) since flavan-3-ols have the band shifted more to longer wavelengths >350 nm.
419	Visible (violet)	This may originate from chlorophyll and carotenoids. Contribution of chlorophyll to this peak could be assessed based on the observed absorption in the red region where carotenoids do not absorb. Anthocyanins are very pH sensitive, usually flavonoids (and anthocyanins) move their longer-wavelength absorption band to higher wavelengths (hypsochromic shift), in acidic extracts- i.e., the absorption by anthocyanins will be shifted to longer wavelengths and would be better assessed using 50% aqueous methanol extracts.
656	Visible (red)	Likely to originate from chlorophyll

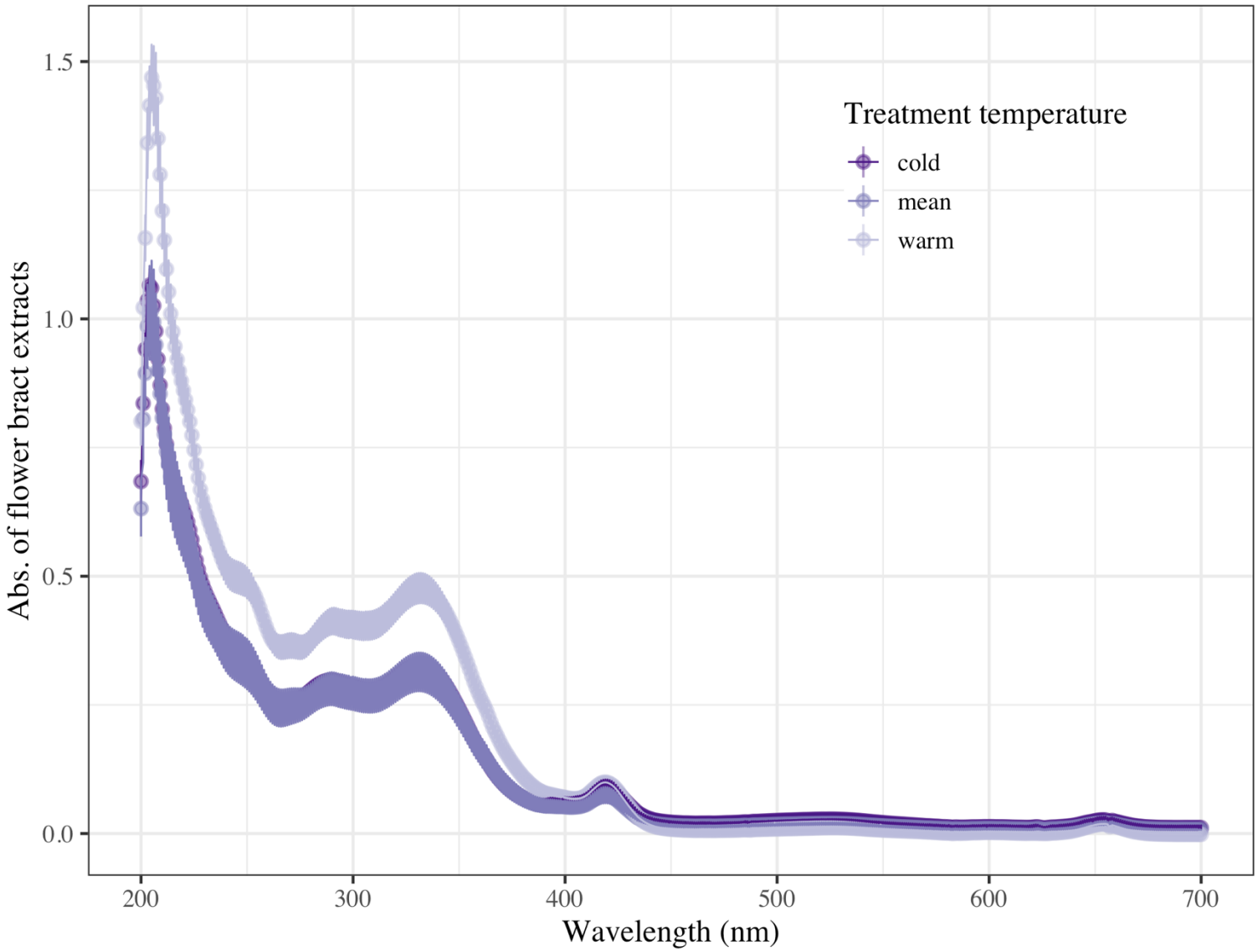

Figure 9. Mean absorbance of flower bract extracts at each wavelength (nm) for each treatment temperature.

4. Discussion

4.1 Higher phenolic absorbance of flowers was found at cooler temperatures

The ratio of absorbance of phenolic-compound extracts from the floral bracts in the UV-B to absorbance in the UV-A region was found to be highest in colder temperatures. The peak at 291 nm may partly, but not exclusively, reflect the presence of hydroxy-cinnamic acids (HCA's). Whereas the peak at 331 likely delineates flavonoids. Of these, flavin-based derivatives could be more abundant than flavan-3-ols, since flavan-3-ols have an absorbance band shifted more towards longer wavelengths over 350 nm (Mabry et al. 1970).

Plants synthetize flavonoids, which are a group of phenolic compounds, in response to several biotic and abiotic factors (Chalker-Scott 1999; Falcone Ferreyra et al. 2012). There are many flavonoid groups that serve different functions depending on environmental, ecological, and developmental conditions (Winkel-Shirley 2001; Treutter 2006). Hence, the composition of individual phenolic compounds can be affected by an assortment of selective pressures in nature. For this reason, the diverse functions of flavonoids can lead to correlations between floral color and response to herbivory, which makes functional analysis more challenging.

The observed effect is likely due to a change in the quantity and type of individual phenolic compounds, particularly, flavonoids and HCA's. The ratio of absorbance at 291 nm and 331 nm differs according to the relative abundances of different classes of phenolic compounds present. Changing the ratio of different phenolic compounds results in a change to the peak sizes in the UV regions of the whole extract absorption spectrum that reflects the relative composition of these compounds, since peaks represent the sum of all compounds present. In other words, a shift in composition within a class of compounds, such as flavonoids, can lead to slightly different functions and chemical structures, which influence how well the floral bracts absorb light. This shift would imply a functional difference, for instance, a shift between those flavonoids that principally function to shield the interior of the flower from UV radiation, and those which function mainly as antioxidants reducing general stress from reactive oxygen species (ROS), which can be due to many factors including temperature, UV radiation, light, and biotic stress (Treutter 2006; Bautista et al. 2016). Phenolic compounds, predominantly flavonoids, respond well to such stressors because they are antioxidants and efficient ROS scavengers (Bautista et al. 2016).

Conversely, while hydroxycinnamic acids are also efficient ROS scavengers, they also function as structural components of cell walls (Tattini et al. 2004).

Small shifts in the biosynthetic pathway can lead to different spectral absorbances, for instance, Albert et al. (2009) found that an increase in the ratio of phenolic metabolite quercetin compared to kaempferol flavanols resulted from a 5°C decrease in temperature. UV-B radiation (280-315 nm) can cause damage to plant cells, so epidermally located hydroxycinnamic acid derivatives and flavonoids can also serve to protect plant cells against predominately UV-B and UV-A radiation, respectively (Bilger et al. 2007). Flavonoid content of plants has also been known to vary across environmental gradients, including latitudinal gradients (Nybakken et al. 2004) and temperature gradients (Lacey et al. 2010; Lacey et al. 2012). Bautista et al. (2015) noticed a significant positive correlation between total phenolic and antioxidant flavonoid content increasing with higher temperatures in 19 wild plant species, this change in phenolic absorbance related to growing temperature is consistent with that reported here.

Another possible explanation of the change in peak ratios reported here is that the observed effect may be caused by a different flavonoid to the hydroxy-cinnamic acid ratio. Namely, a shift earlier in the biosynthetic pathway, whereby more precursors are channeled towards one group of compounds, for example, flavonoids, and therefore less go to HCA's. Consequently, temperature changes can produce changes in gene expression and enzymatic activity affecting the biosynthetic pathway, which may have led to the type of changes in absorbance observed in this study. Contrastingly, del Valle et al. (2015) observed a positive relationship between anthocyanins and other groups of flavonoids in *Silene littorea*, signifying that the biosynthetic pathway leading to the accumulation of these compounds can be generally upregulated in the same way rather than always involving a trade-off of different classes of compounds against one another.

Changes in composition of the phenolic profile with temperature treatment, resulted in both higher and lower peaks of absorbance differing across the regions of the spectrum. Peak ratios were analyzed rather than peak absorbance to account for potentially non-linear differences in absorbance at different dilutions. Nevertheless, when plotting mean peak absorbance for each treatment against each other, the spikes in the warm treatment visually had greater phenolic absorbance compared with the mean and cold treatments. However, absorbance was expected to be greater under cooler conditions. If there was greater absorbance of sunlight by spikes under cooler conditions, this should also be visible in the reflectance results. Explanations for why there

would be more absorbance under warm temperatures could be either functional or metabolic. A metabolic explanation would be that the plant grew better in warmer temperatures, so it had more excess resources to allocate to phenolic compounds. This explanation is partly evidenced by heavier flower spikes being identified in warmer temperatures, meaning there were excess resources in the warm treatment compared to the mean and cold treatments. A functional explanation would be that the plant would associate warmer temperatures with more sunlight during the flowering period, and so these compounds that can protect from damage by high solar irradiance would be upregulated. Determining whether the phenolic absorbance is explained by a functional or metabolic response is beyond the scope of this study but could be tested through an experimental manipulation which disassociated temperature and solar radiation and measured the carbon assimilation and allocation to phenolic metabolite of these plants. In summary, consistent with previous results on the mechanisms behind flower color (Stiles et al. 2007; Lacey et al. 2010; Anderson et al. 2013; Berardi et al. 2016; Bautista et al. 2016), phenolic absorbance in flower bracts strongly varied with temperature. The results presented in this study provide an improved understanding of anthocyanin production in a northern population of *P. lanceolata*.

4.2 Spike reflectance in the IR spectrum increased with growing temperature of plants

Spike reflectance in the NIR region was found to be temperature sensitive, thus, expressed thermoregulatory plasticity. The results presented here are in agreement with other studies of *P. lanceolata* floral traits (Lacey and Herr 2005; Stiles et al. 2007; Lacey et al. 2010; Anderson et al. 2013), in that reflectance in the IR spectrum increased with rising temperature. Lacey and Herr (2005) established that reflectance in the visible and IR spectrum is plastic at the individual plant level, constituting a thermoregulatory reaction to changes in ambient temperature. The plants in the cold treatment in this study produced darker flowers, that could absorb more solar radiation, compared with lighter flowers in the warm treatment. One explanation for decreased reflectance in the IR spectrum in colder temperatures could be that floral bracts increase their absorbance to raise the temperature of their floral reproductive tissues (Lacey and Herr 2000). Considering the ideal radiation-absorbing shape of *P. lanceolata* floral spikes, which are cylindrical and opaque, solar radiation is absorbed better by less reflective spikes and this additional warmth could be conducted to the tissue within them. Spikes that absorb more solar radiation can keep their gametes, gametophytes, and embryonic progeny warm during cool periods of the growing season. This has been shown to improve offspring fitness (Lacey and Herr 2000).

Northern populations of *P. lanceolata* have been found to display a greater thermoregulatory capacity at cool temperatures by reducing their floral reflectance in the IR spectrum (Lacey et al. 2010). The study population was a northern population of *P. lanceolata* (Image 5). Thus, the population on Åland likely demonstrates a high thermoregulatory capacity, which offers another possible explanation as to why decreased absorbance was identified in cooler temperatures.

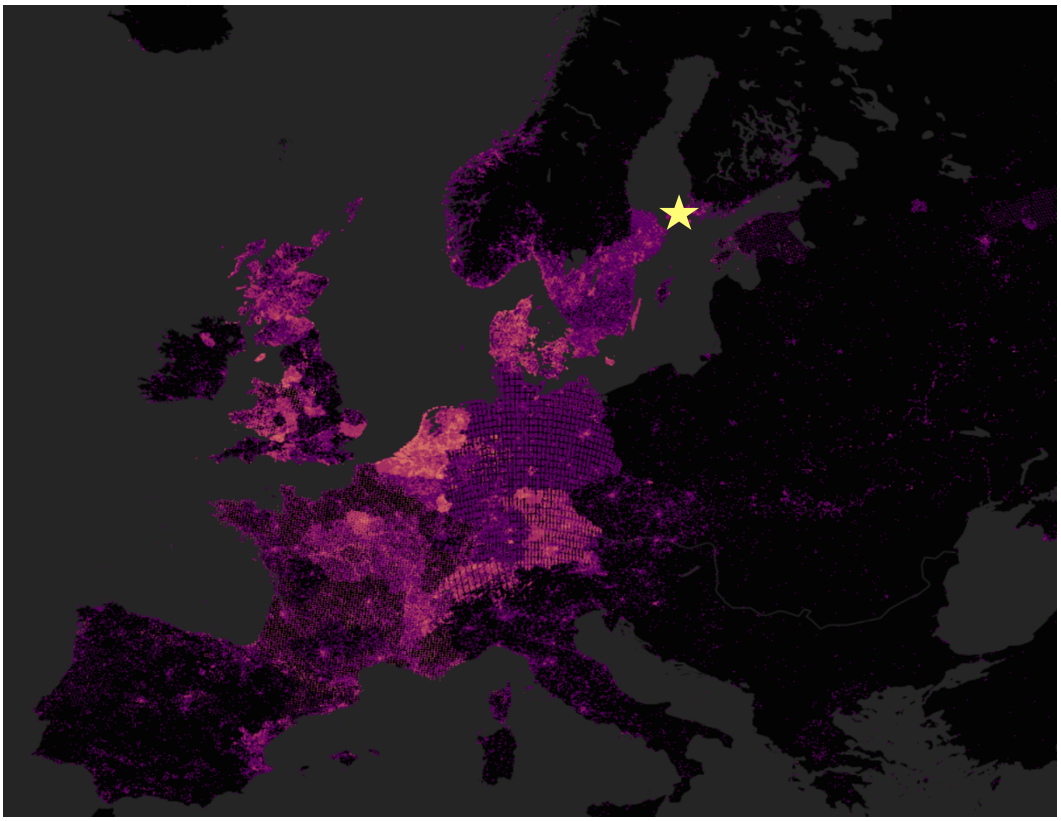

Image 5. European distribution in *P. lanceolata* (orange indicates dense observations of the species, purple indicates scarce observations of the species) showing the location of the study population. Map adapted from *Plantago lanceolata* L. in GBIF Secretariat (2019).

The results of this study reveal that this population of *P. lanceolata* is likely very plastic regarding thermoregulation. Genotypes of *P. lanceolata* that are non-plastic produce only highly reflective spikes, regardless of ambient temperature (Lacey et al. 2010). Individuals from this population produced both high and low reflectance in the IR region of the spectrum, and therefore expressed plasticity in reflectance. Plants expressing thermoregulatory plasticity can alter their phenology to better adapt to a variable climate and thus are able to produce offspring earlier in the

season, which can have a fitness advantage (Lacey and Herr 2005), thus suggesting another explanation of the results.

4.3 Flower spike surface temperature was not influenced by its reflectance in the IR spectrum or phenolic content

Surface temperature of flower spikes was not affected by reflectance or phenolic content, when measured with an IR thermometer. Factors that could have influenced surface temperature measurements include evapo-transpiration, flower size, water content, and the emission spectrum of the lightbulb used to warm up the spike. Firstly, stomatal control of water loss from the flower may have a larger effect on its temperature through evaporative cooling than its optical properties in controlling its temperature. Additionally, the mass of the flower determines how quickly it equilibrates with the ambient temperature, so spike size could affect how quickly it adjusted to the heat given off by the lamp (heat source). Furthermore, water absorbs in a broad region across the infra-red, so if the wavelengths of measurements from the IR thermometer coincide with these wavebands, then the water content of the flower could have a larger effect than its coloration. Moreover, the bulb emits more light at a longer wavelength than the plant would receive in nature, so there could be a mismatch between adaptation to the natural solar spectrum in the environment and in controlled conditions, which could mask effects on surface temperature from the IR thermometer.

Previous studies have identified an effect of flower reflectance on surface temperature (Lacey and Herr 2005). The method used in this study to measure spike temperature was different from the method used in Lacey and Herr (2005). In their study, Lacey and Herr (2005) measured internal spike temperature outdoors in full sun and used a thermocouple wire, which was inserted into each spike to record the temperature within. Moreover, Lacey and Herr (2005) reported technical difficulties of measuring internal flower temperature outdoors and observed that sunlight warmed low-temperature clones more than it did greenhouse clones. When wind was present in their experiment, temperature differences declined. Lacey and Herr (2005) reported that the effect of reflectance on internal temperature of spikes is still unclear. Differences in measurements, including indoor versus outdoor measurements, and surface temperature measurements versus internal spike temperature measurements, can partially rationalize the variation in results. Thus, there are several drawbacks of both spike temperature measurement techniques; neither are invalid but should be treated with caution.

5. Conclusion

This study shed further light on thermoregulatory plasticity, with meaningful implications for plant responses to climate warming. The results suggest that the Åland population of *P. lanceolata* can utilize thermoregulatory plasticity to adjust to rising temperatures by adjusting flower bract reflectance in the IR region and phenolic absorbance. This study provided more insight into temperature regulation of anthocyanin production in a northern population of *P. lanceolata.* Increased selection for thermal functional traits may occur in future climate scenarios of continued warming in temperate and boreal biomes. However, the exact mechanisms driving changes to reflectance, phenolic absorbance, and surface temperature need more investigation. Among these possible drivers, are (1) increased absorbance by floral bracts to counteract cold temperatures and protect embryonic progeny; and (2) a shift in ratios of phenolic compounds to combat biotic and abiotic stress. The effects of these strategies should be visible as changes in flower temperature, but an improved method of measuring the surface temperature of spikes is firstly needed. The primary factor that explained reflectance and phenolic absorbance was ambient temperature, nevertheless there are likely numerous factors that contribute to reflectance and phenolic absorbance of flowers, which are beyond the framework of this study. The next step in research would be to compare current populations to past populations by means of herbaria specimen to determine if floral traits, such as pigmentation, have changed over time. To achieve this, one could grow plants from viable herbaria specimen seeds to compare with freshly harvested seeds and set up a greenhouse experiment with different temperature groups reflecting past, present, and future climate scenarios. This would lay the foundation for a more accurate understanding of the rate of plasticity as climate warms.

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I would like to thank my master's thesis supervisors Maria Hällfors and Matthew Robson for their advice, expert guidance, and constructive evaluations throughout the whole process. Through working with Maria, I have learned to think critically and take a "big picture" approach to science, while at the same time setting boundaries and learning to take breaks. She is a great female scientist and role model. Matt has introduced and inspired me to learn more about plant ecophysiology, and always made sure I was aware of every learning and networking opportunity available. I would also like to thank Pedro Aphalo for letting me use his camera and offering me guidance with

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8. Appendix

Table S1. Origins of populations from Åland (Coordinates are in the WGS84 projection; sample individuals refer to how many individual's seeds were sampled from).

Population	Latitude (°N)	Longitude (°E)	Location	Municipality	Sample individuals
A	60.06770	20.071867	Söderby	Lemland	50
B	60.13275	19.989163	Österkalmare	Jomala	50
C	60.17423	19.523903	Skeppsvik	Eckerö	50
D	60.22434	19.559542	Storby	Eckerö	28
E	60.27522	20.240213	Hulta	Sund	17
F	60.25415	20.228615	Mångstekta	Sund	39
G	60.26042	20.178783	Strömbolstad	Sund	26
H	60.08355	19.906516	Gregersö, Möckelö	Jomala	20
I	60.06605	19.951663	Espholm, Ytternäs	Maarienhamina	48

Table S2. Full model (using syntax from the lmer function as implemented in the lme4 R-package (Bates et al. 2015)) for each response variable.

Response	Full model
Reflectance in IR	~ Treatment temperature + (1|Population) + (1|Replicate)
Phenolic abosrbance (291:331 nm)	~Treatment temperature + (1|Population=!"A")
Surface temperature of spike	~Phenolic abosrbance (291:331 nm) + (1|Population)
Surface temperature of spike	~Reflectance in IR + Treatment temperature + (1|Population) + (1|Replicate)
Dry weight of spike	~ Treatment temperature + (1|Replicate)

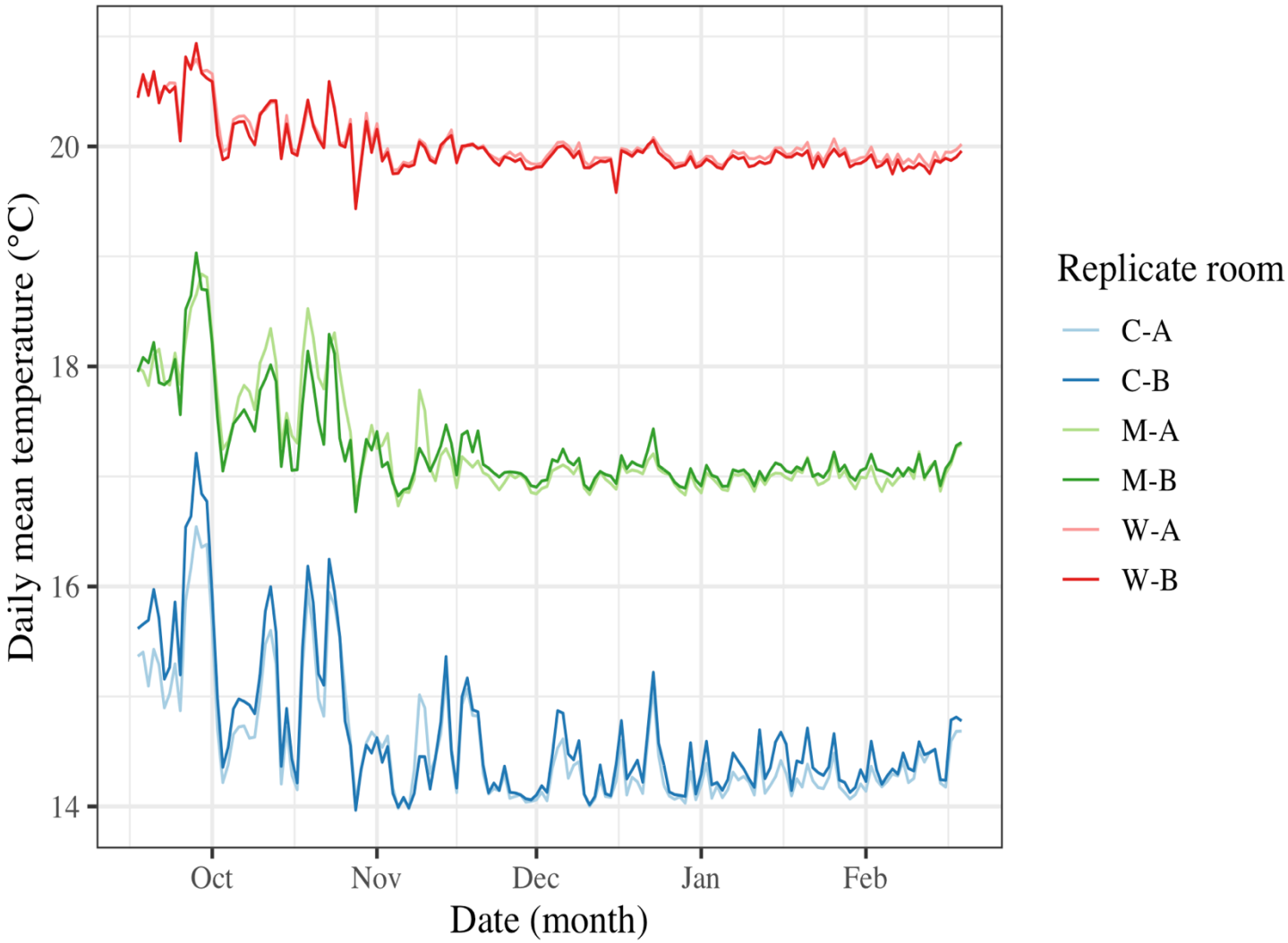

Figure S1. Daily mean temperatures (°C) during the duration of the experiment.

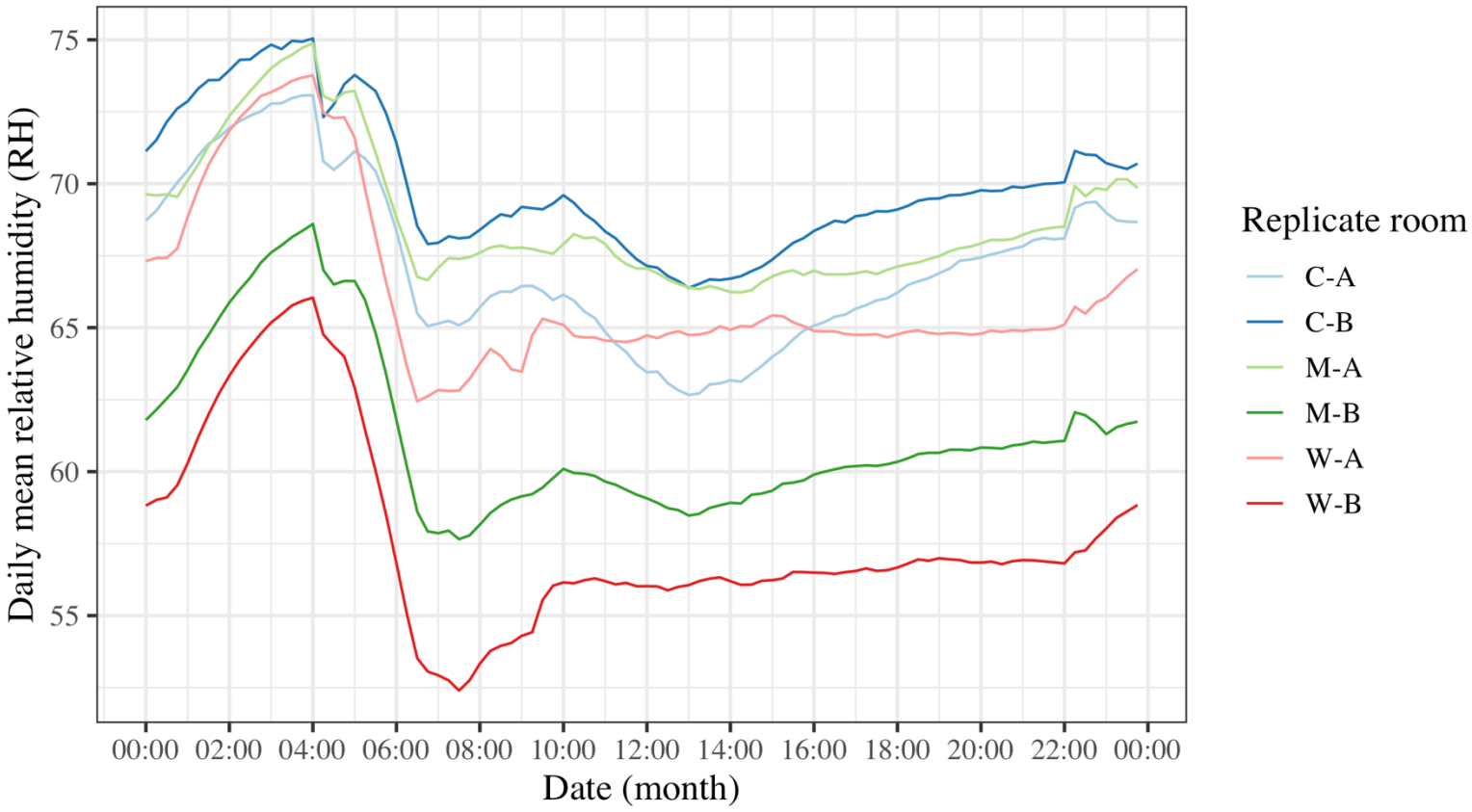

Figure S2. Room humidity (RH) means over a 24-hour period, taken at 15-minute intervals from 17.09.2019 – 19.02.2020.

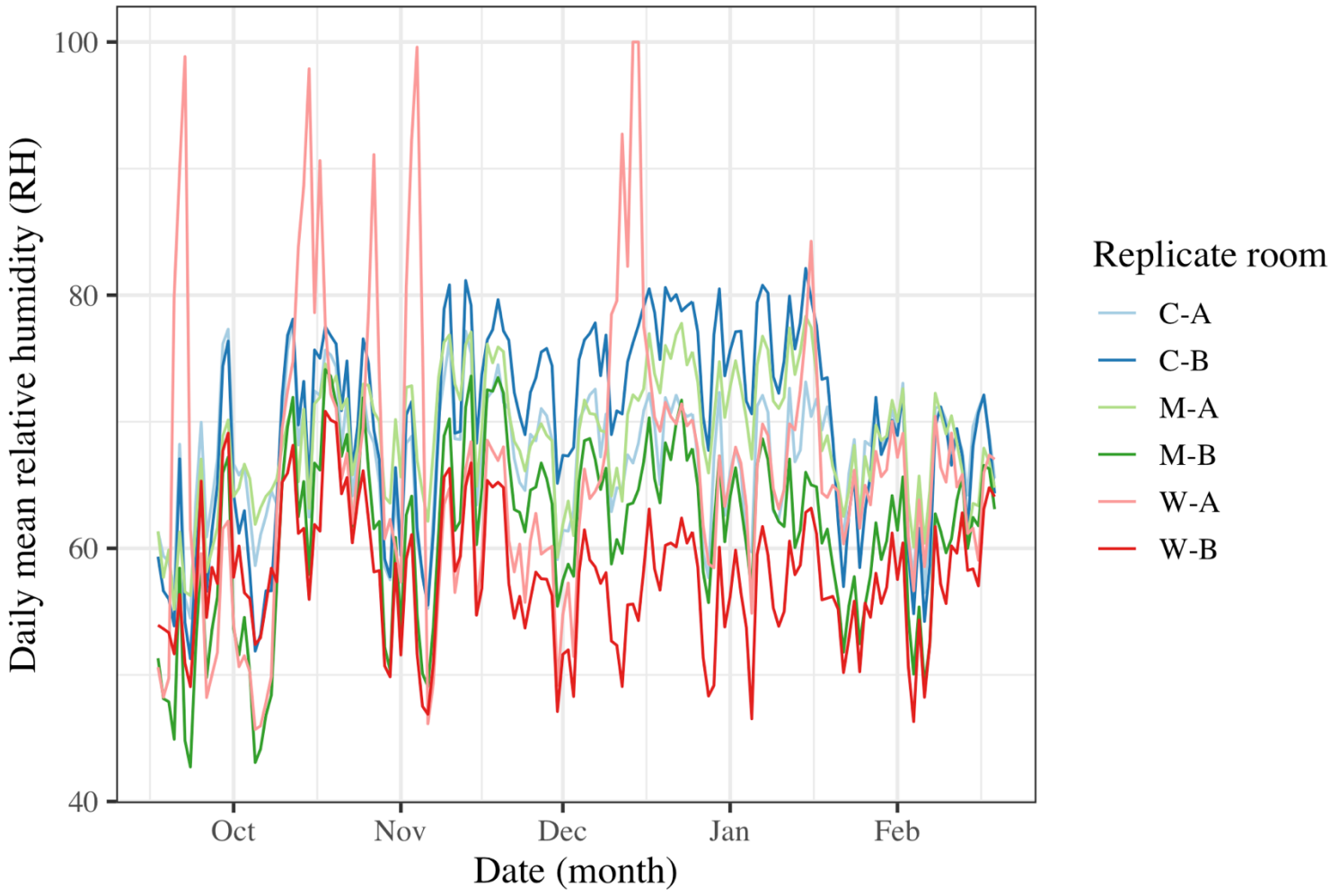

Figure S3. Daily mean humidity (RH) during the duration of the experiment.

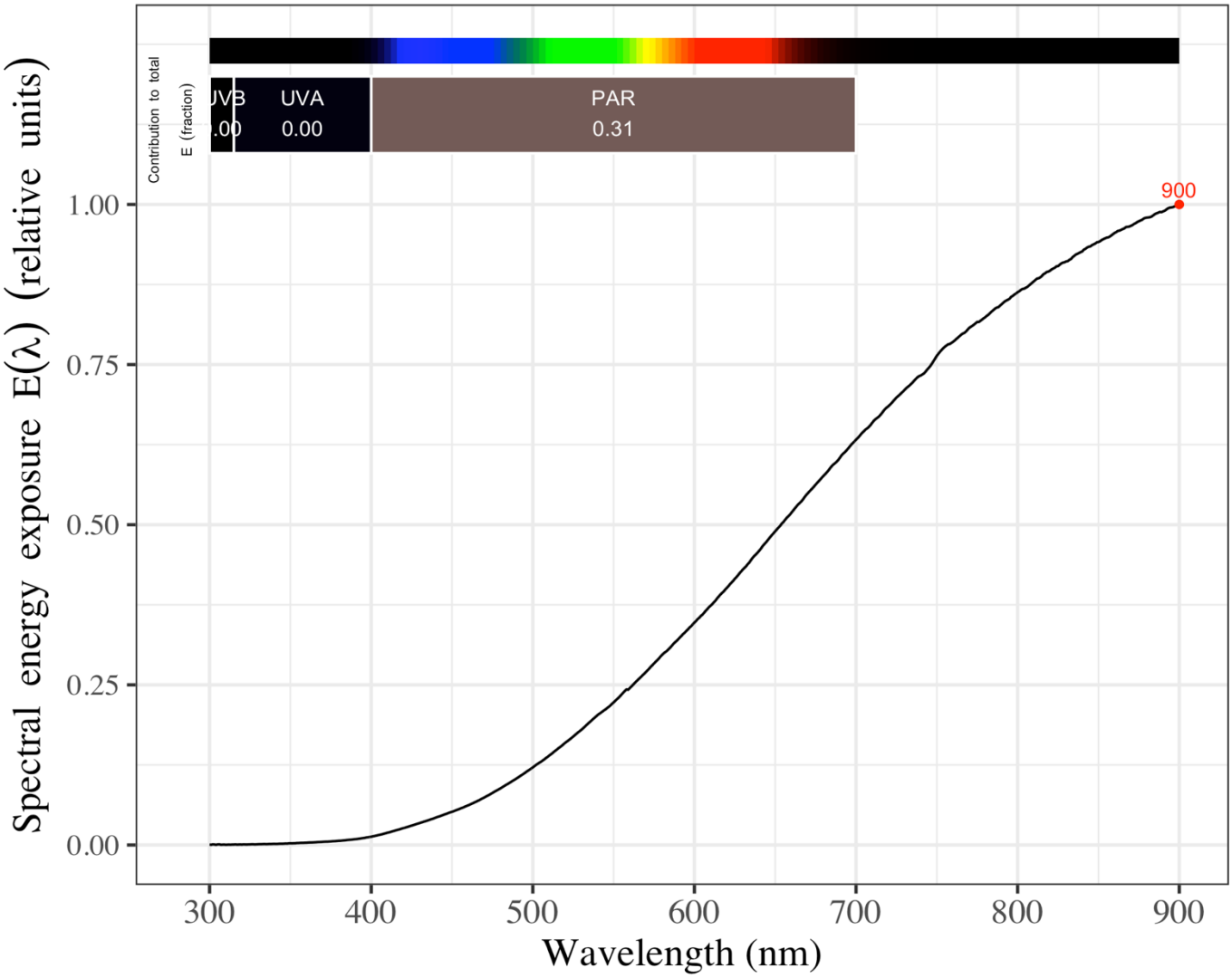

Figure S4. Wavelength (nm) of spectral energy exposure for incandescent 60w bulb used as light (heat) source for surface temperature recordings and light source for IR image capture.

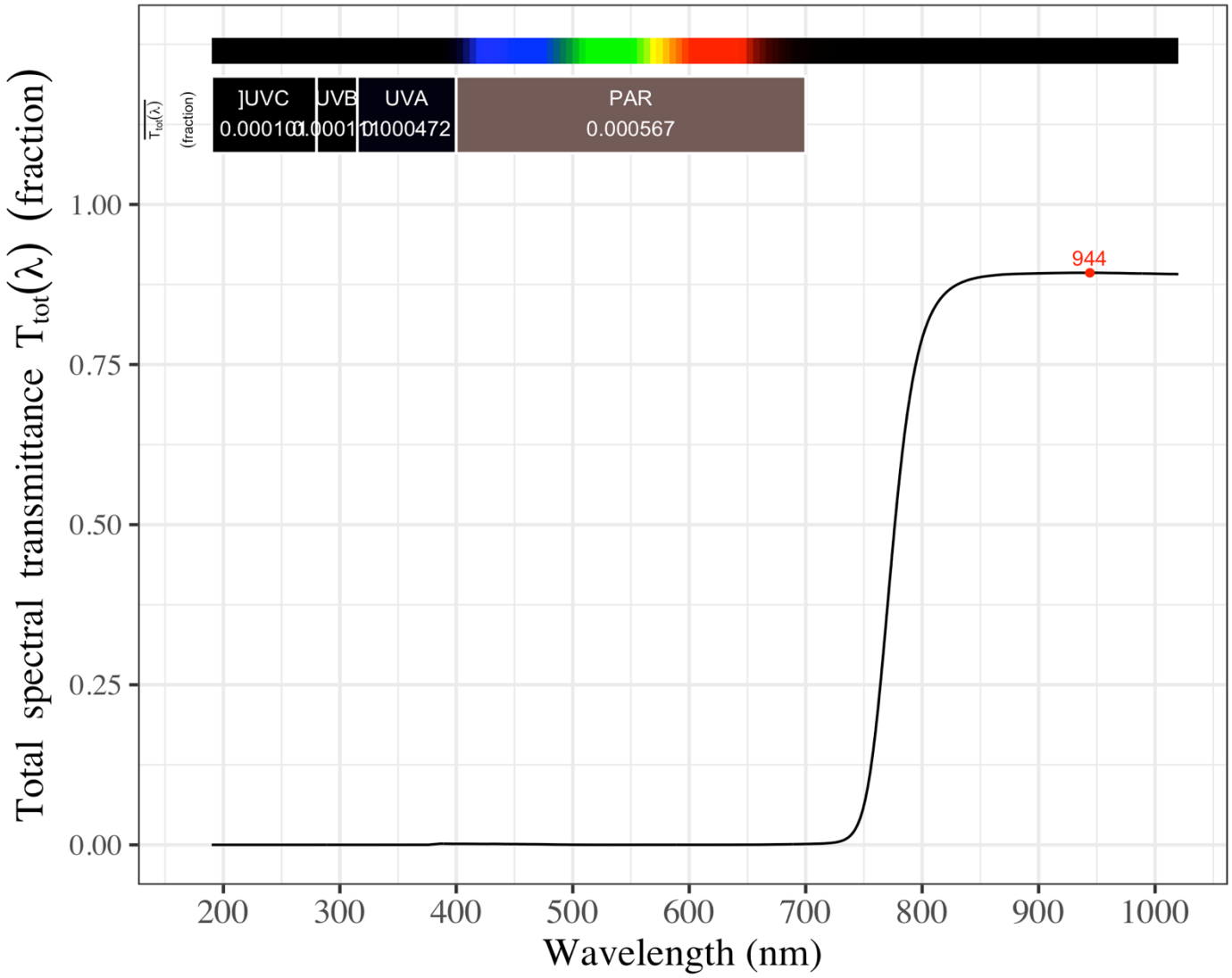

Figure S5. Wavelength (nm) of spectral energy exposure for the Heliopan RG780 ES 52 filter, which attenuates light before 780 nm.